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FoxO3 Transcription Factor and Sirt6 Deacetylase Regulate Low Density Lipoprotein (LDL)-cholesterol Homeostasis via Control of the Proprotein Convertase Subtilisin/Kexin Type 9 (*Pcsk9*) Gene Expression*

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Background: PCSK9 is critical for LDL-cholesterol regulation, but the epigenetic regulation of the *PCSK9* gene is not clear.
Results: FoxO3 and Sirt6 suppress the *PCSK9* gene expression and reduce LDL-cholesterol.
Conclusion: Hepatic FoxO3 and Sirt6 control LDL-cholesterol homeostasis.
Significance: FoxO3 and Sirt6 are important for cardiovascular health.

Elevated LDL-cholesterol is a risk factor for the development of cardiovascular disease. Thus, proper control of LDL-cholesterol homeostasis is critical for organismal health. Genetic analysis has identified *PCSK9* (proprotein convertase subtilisin/kexin type 9) as a crucial gene in the regulation of LDL-cholesterol via control of LDL receptor degradation. Although biochemical characteristics and clinical implications of PCSK9 have been extensively investigated, epigenetic regulation of this gene is largely unknown. In this work we have discovered that Sirt6, an NAD^+-dependent histone deacetylase, plays a critical role in the regulation of the *Pcsk9* gene expression in mice. Hepatic Sirt6 deficiency leads to elevated *Pcsk9* gene expression and LDL-cholesterol as well. Mechanistically, we have demonstrated that Sirt6 can be recruited by forkhead transcription factor FoxO3 to the proximal promoter region of the *Pcsk9* gene and deacetylates histone H3 at lysines 9 and 56, thereby suppressing the gene expression. Also remarkably, overexpression of Sirt6 in high fat diet-fed mice lowers LDL-cholesterol. Overall, our data suggest that FoxO3 and Sirt6, two longevity genes, can reduce LDL-cholesterol levels through regulation of the *Pcsk9* gene.

Elevated LDL-cholesterol is a risk factor for cardiovascular disease (1). High LDL-cholesterol can be caused by a number of dysregulated processes, including increased cholesterol biosynthesis, increased VLDL secretion, and decreased LDL clearance (2). Genetic studies have identified mutations in at least three genes that significantly contribute to autosomal dominant hypercholesterolemia, and they are LDL receptor (*LDLR*),[2] apolipoprotein B (*APOB*), and proprotein convertase subtilisin kexin type 9 (*PCSK9*) (3). LDLR plays a major role in the LDL clearance. Apolipoprotein B, a protein component of LDL, also interacts with LDLR. PCSK9 can modulate the LDL metabolism through control of the LDLR degradation in the lysosome (3).

Since the discovery of PCSK9 mutations in the autosomal dominant hypercholesterolemia patients a decade ago (4), significant progress has been made in the understanding of PCSK9 biochemistry and pathophysiology (5). Now we know that PCSK9 is expressed mainly in the liver as a ~72-kDa precursor and can be auto-cleaved in the endoplasmic reticulum to an ~62-kDa mature form that is secreted to plasma. Circulating PCSK9 binds to the extracellular EGF-A domain of the LDLR and targets it for degradation in the lysosome (5). The physiological function of PCSK9 in the control of LDL-cholesterol has also been confirmed by mouse genetics. Overexpression of PCSK9 in mice leads to hypercholesterolemia, and the *Pcsk*9 gene knock-out in mice dramatically reduces LDL-cholesterol (6–13). Because of this biological function, PCSK9 has become a useful target for lowering LDL-cholesterol, and several clinical trials are in progress to validate the efficacy of targeting PCSK9 for cardiovascular disease (14–21).

PCSK9 gene expression can be induced by insulin and pioglitazone and also can be suppressed by glucagon, bile acids, berberine, fibrate, and oncostatin M (22–32). PCSK9 protein levels decrease in the course of fasting and increase after feeding (22, 27, 29, 32–34). A number of transcription factors or cofactors have been shown to regulate the PCSK9 gene expression, including sterol-response element binding proteins (SREBP-1/2), hepatocyte nuclear factor 1A (HNF1A), farnesoid X receptor, peroxisome proliferator-activated receptor γ, liver X receptor, and histone nuclear factor P (24, 28, 29, 33–37). However, how the *PCSK9* gene expression is controlled by epigenetic

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[2] The abbreviations used are: LDLR, LDL receptor; HFD, high fat diet; HNF1A, hepatocyte nuclear factor 1A; Mttp, microsomal triglyceride transfer protein; PCSK9, proprotein convertase subtilisin kexin type 9; SIRT6, sirtuin 6; SRE, sterol response element; SREBP, sterol-response element binding protein; IP, immunoprecipitation.

chromatin remodeling is not clear. In this work we have identified sirtuin 6 (SIRT6) as a critical histone deacetylase for the *PCSK9* gene regulation and LDL-cholesterol homeostasis.

MATERIALS AND METHODS

Animal Studies—FoxO1 (forkhead box O1), FoxO3, FoxO1/3/4, Sirt1, and Sirt6 liver-specific knock-out mice were produced by crossing floxed mice with an albumin-Cre line from The Jackson Laboratory. Animals were maintained on the following genetic background: FoxOs floxed mice on C57BL/6J:129/Sv:FVB, Sirt1 floxed mice on C57BL/6J:129/Sv, and Sirt6 floxed mice on NIH Black Swiss:129/Sv:FVB. Genotyping was carried out as previously described (38–40). High fat diet (60% calories from fat) was purchased from Harlan Laboratories (Madison, WI). For the VLDL secretion analysis, mice were fasted for 4 h before a dose of 500 mg/kg body weight Triton WR1339 was injected via tail vein. Blood samples were collected and analyzed as previously described (41). All animal procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Use and Care Committee of Indiana University School of Medicine.

Plasmid Constructs and Adenoviruses—For mouse *Pcsk9* gene promoter analysis, we cloned the short promoter (−128 to +330 bp relative to the transcriptional start site) together with the 5′-untranslated region (UTR) into pGL4.10 vector (Promega) using the primers mPcsk9-pro-forward (5′-GGCCCAGGAGAAGTTAGTTAATA-3′) and mPcsk9-pro-reverse (5′-ATGTCTCTGGGGAGCCAA-3′). Human FOXO3 and SIRT6 and mouse HNF1A coding sequences were cloned into pcDNA3 (Invitrogen) with a FLAG or HA tag. Adenoviruses for SIRT6 and FOXO3 overexpression were generated as previously described (39, 42). Mouse Pcsk9 shRNAs were designed using the BLOCK-iT RNAi Designer (Invitrogen), and DNA oligos were cloned into a pENTR/U6 vector for further adenovirus generation. The sequence of the Pcsk9 shRNA used in this work is: 5′-CAGGACGAGGATGGAGATTAT-3′. For Sirt6 gene overexpression *in vivo*, adenoviruses were injected into mice via tail vein at a dose of 5×10^8 pfu.

Serum and Liver Cholesterol Analysis—Blood samples were collected from overnight-fasted mice. Hepatic lipids were extracted as previously described (39). Total cholesterol and HDL- and LDL/VLDL-cholesterol were analyzed using assay kits from Wako Chemicals USA.

Luciferase Reporter Assays—Mouse *Pcsk9* gene promoter (also including 5′-UTR) was analyzed in HEK293 cells using the pGL4.10 luciferase reporter system together with an internal control Renilla luciferase reporter as previously described (39).

mRNA Analysis—Total RNAs were isolated from cells and tissues using TRI Reagent (Sigma). Reverse transcription was performed using a cDNA synthesis kit (Applied Biosystems). Real-time PCR was performed using GoTaq qPCR Master Mix (Promega). The primers used in PCR reactions were as follows: mMttp forward, 5′-ATGATCCTCTTGGCAGTGCTT-3′; mMttp reverse, 5′-TGAGAGGCCAGTTGTGTGAC-3′; mPcsk9 forward, 5′-GGAACCTGGAGCGAATTAT-3′; mPcsk9 reverse, 5′-CACCCTGGATGCTGGTATCT-3′; mSirt6 forward, 5′-ACGTCAGAGACACGGTTGTG-3′; mSirt6 reverse, 5′-CCTCTACAGGCCCGAAGTC-3′. Real-time PCR data were normalized to an internal control; Ppia and relative -fold changes (experimental group/control) were also calculated.

Protein Analysis—Cell and tissue extract preparation, immunoprecipitation, and immunoblotting were performed as described previously (39). The following antibodies were used: anti-actinin, anti-HA, anti-FoxO1, anti-FoxO3, anti-acetylated lysine (Cell Signaling Technology), anti-LDLR and anti-Pcsk9 (Cayman Chemical), anti-HNF1A (Santa Cruz Biotechnology), and anti-FLAG and anti-SIRT6 (Sigma).

Chromatin Immunoprecipitation (ChIP)—Chromatin association analysis was performed in mouse primary hepatocytes and mouse livers followed by chromatin preparation, immunoprecipitation with FLAG (Sigma), HA (Cell Signaling Technology), H3K9Ac, H3K56Ac, and histone H3 (Millipore) antibodies and the endogenous protein antibodies described above and real-time PCR analysis, as described previously (39). ChIP DNA amount for gene promoters of interest was normalized to that of a housekeeping gene, Ppia ChIP or total histone H3 ChIP. Primers used in the ChIP PCR reactions were: mPcsk9-ChIP forward, 5′-CGAAACCTGATCCTTTAGTACC-3′; mPcsk9-ChIP reverse, 5′-ATGTCTCTGGGGAGCCAA-3′; mPpia-ChIP forward, 5′-CAGACCCACATTCCTGAGGT-3′; mPpia-ChIP reverse, 5′-AAGTCGGTGCTGTGGAAGAC-3′.

Statistical Analysis—Quantitative data were presented as the mean ± S.E. Significance ($p < 0.05$) was assessed by two-tailed unpaired Student's *t* test.

RESULTS

LDL-cholesterol Is Elevated in Hepatic Sirt6-deficient Mice—Sirt6 has been previously shown to regulate hepatic triglyceride metabolism and cholesterol biosynthesis (38, 43). To examine which lipoprotein-associated cholesterol might be modulated by Sirt6, we analyzed cholesterol in HDL and LDL/VLDL fractions of sera from control floxed (LoxpT6) and Sirt6 liver-specific knock-out mice (LKOT6). Whereas there was no significant difference in HDL-cholesterol, LDL/VLDL-cholesterol levels were increased 45% in the LKOT6 mice relative to the control LoxpT6 littermates (Fig. 1, *A* and *B*). VLDL secretion was also increased in the LKOT6 mice compared with the control mice (Fig. 1*C*). Microsomal triglyceride transfer protein (Mttp), an important factor for VLDL assembly and secretion, was moderately up-regulated in the LKOT6 livers (Fig. 1*D*).

Sirt6 Regulates LDL-cholesterol by Suppression of the Pcsk9 Gene Expression—Because hepatic deficiency of Sirt6 led to elevated LDL-cholesterol but not HDL-cholesterol, we decided to further investigate the underlying mechanisms. As Pcsk9 is critically involved in LDLR turnover and LDL-cholesterol homeostasis (5), we first analyzed Pcsk9 mRNA and protein levels in control and LKOT6 livers. The results showed that Pcsk9 mRNA was increased by ~3-fold in the LKOT6 mice as compared with the control mice (Fig. 2*A*). Consistent with an increase in Pcsk9 mRNAs, its protein level was also elevated in the LKOT6 liver (Fig. 2*B*). Because Pcsk9 targets LDLR for degradation, we also observed a decrease in LDLR in the LKOT6 liver (Fig. 2*B*). To verify the role of Pcsk9 in LDLR degradation, we performed *Pcsk9* gene knockdown in mouse primary hepatocytes. As expected, knockdown of Pcsk9 led to a significant

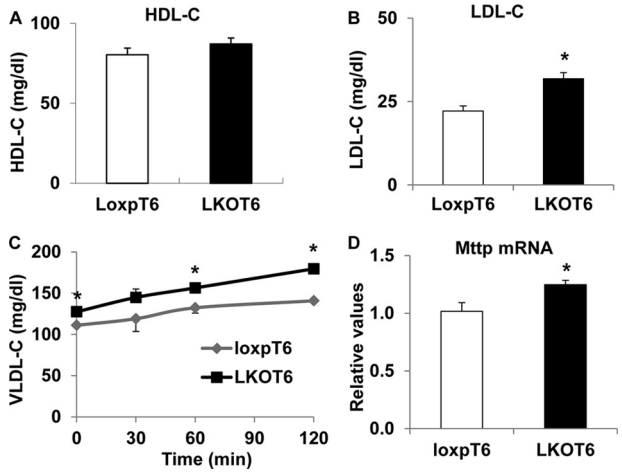

FIGURE 1. **Hepatic Sirt6 knock-out leads to elevated LDL-cholesterol levels.** *A* and *B*, serum HDL- and LDL-cholesterol (*HDL-C* and *LDL-C*, respectively) measurements in 2–3-month-control floxed (*loxpT6*) and *Sirt6* liver-specific knock-out mice (*LKOT6*, $n = 10–12$). *C*, VLDL-cholesterol (*VLDL-C*) secretion analysis in control and LKOT6 mice ($n = 3–4$). *D*, real-time PCR analysis of Mttp mRNA in the liver of control and LKOT6 mice ($n = 6–10$). Data are the mean ± S.E.; *, $p \leq 0.05$ by *t* test.

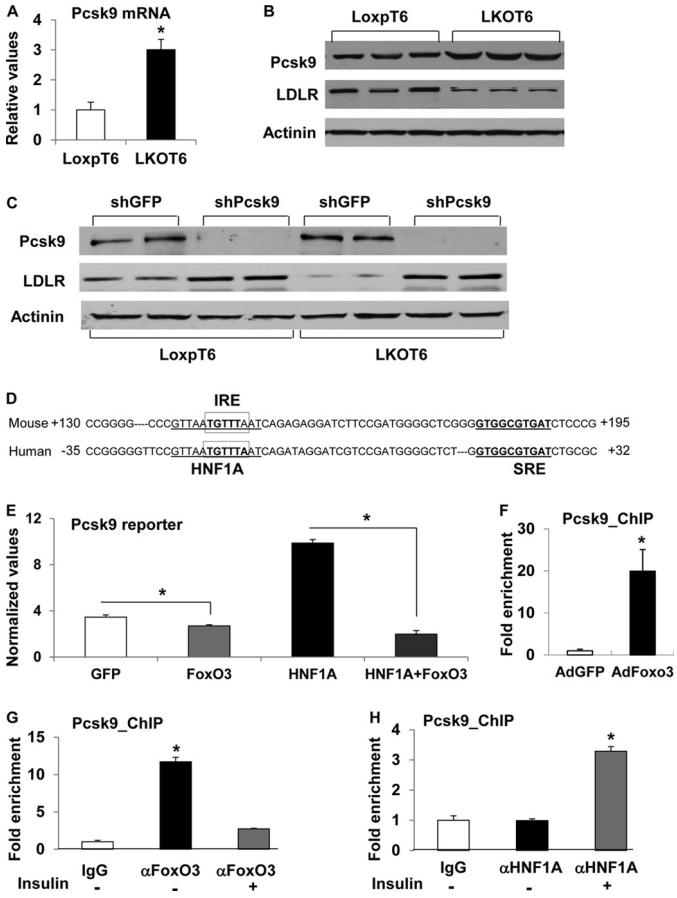

FIGURE 2. **Regulation of the *Pcsk9* gene by FoxO3 and Sirt6.** *A*, real-time PCR analysis of Pcsk9 mRNA in the control and LKOT6 livers ($n = 3–5$). *B*, immunoblot analysis of Pcsk9 and LDLR proteins in the control and LKOT6 livers. *C*, immunoblot analysis of Pcsk9 and LDLR proteins in mouse primary hepatocytes infected with control *GFP* (*shGFP*) and *Pcsk9* (*shPcsk9*) shRNA adenoviruses, respectively. *D*, *Pcsk9* gene promoter analysis. A potential FoxO binding element also called insulin response element (*IRE*, *inside the box*) was identified in the UTR of mouse *Pcsk9* gene and human *PCSK9* proximal gene promoter. Sequence numberings refer to the transcriptional start site. The previously characterized HNF1A and SREBP2 binding elements are *underlined*, respectively. *E*, luciferase reporter analysis of the proximal promoter (including part of the 5′-UTR) of the mouse *Pcsk9* gene was performed in HEK293 cells that were transfected with respective promoter constructs with GFP, HNF1A, and FoxO3. *F*, analysis of FoxO3 association with the 5′-UTR of the *Pcsk9* gene was performed using ChIP in mouse primary hepatocytes transduced with GFP or FoxO3-expressing adenoviruses. Data are shown as -fold enrichment relative to GFP. *G* and *H*, ChIP analysis of the association of FoxO3 and HNF1A with the 5′-UTR of the *Pcsk9* gene in mouse primary hepatocytes in the absence or presence of 2 nM insulin using control IgG or corresponding protein antibodies. Data are presented as -fold enrichment relative to the IgG control. Data are the mean ± S.E.; *, $p \leq 0.05$ by *t* test.

increase in the LDLR proteins in both wild-type and LKOT6 hepatocytes (Fig. 2*C*).

To explore the regulatory mechanism for the *Pcsk9* gene by Sirt6, we first analyzed promoter sequences of human and mouse *Pcsk9* genes. In addition to previously identified two cis-elements, sterol response element (SRE) and HNF1A binding site, we also found a consensus binding site for FoxO transcription factors (also called insulin response element (*IRE*)) (Fig. 2*D*). Interestingly, the insulin response element is completely embedded in the HNF1A site. This raised a question of whether FoxOs could affect HNF1A-activated *Pcsk9* gene expression. We performed luciferase reporter assays for the proximal promoter region of mouse *Pcsk9* gene, which also includes a part of the UTR containing the HNF1A binding site. To emphasize here, our nucleotide numbering (relative to the transcription start site) is different from the literature because most previous reports have numbered the *Pcsk9* promoter constructs relative to the translation start site. The reporter assay data showed that HNF1A activated the reporter and FoxO3 suppressed the activation by HNF1A (Fig. 2*E*). To verify that FoxO3 is associated with this region in the chromatin, we also performed a ChIP analysis. The data revealed a strong association between the 5′-UTR of the *Pcsk9* gene and FoxO3 (Fig. 2*F*). Interestingly, although insulin reduced the association of FoxO3 to the 5′-UTR of the *Pcsk9* gene, the association of HNF1A was increased (Fig. 2, *G* and *H*).

To further demonstrate that FoxO3 indeed regulates the *Pcsk9* gene expression, we analyzed Pcsk9 mRNA and protein in the livers that were deficient in FoxO1, FoxO3, or FoxO1/3/4 (LKO1, LKO3, and LTKO, respectively). The data indicated that knock-out of FoxO3 led to a significant increase in the *Pcsk9* gene expression in the LKO3 livers, although Sirt6 mRNA levels were not significantly changed (Fig. 3, *A–C*). As a result, hepatic LDLR protein was decreased in the liver of LKO3 mice relative to control mice (Fig. 3*C*). To confirm the correlation between Pcsk9 and LDLR, we also performed *Pcsk9* gene knockdown in control and LKO3 mouse primary hepatocytes. As anticipated, LDLR protein levels were increased after the *Pcsk9* gene was knocked down (Fig. 3*D*). Similar to LKOT6 mice, LKO3 and LTKO mice also had elevated LDL-cholesterol without any significant change in HDL-cholesterol (Fig. 3, *E–H*).

Sirt6 Interacts with FoxO3 and Modulates Histone Acetylation in the Pcsk9 Gene—Because Sirt6 is an NAD-dependent histone deacetylase (44–47), it might be recruited to the *Pcsk9* gene promoter through a transcription factor. We tested this hypothesis by performing analysis of possible protein-protein interactions between Sirt6 and HNF1A, FoxO3, or SREBP-2 by co-immunoprecipitation (co-IP). Our data showed that Sirt6

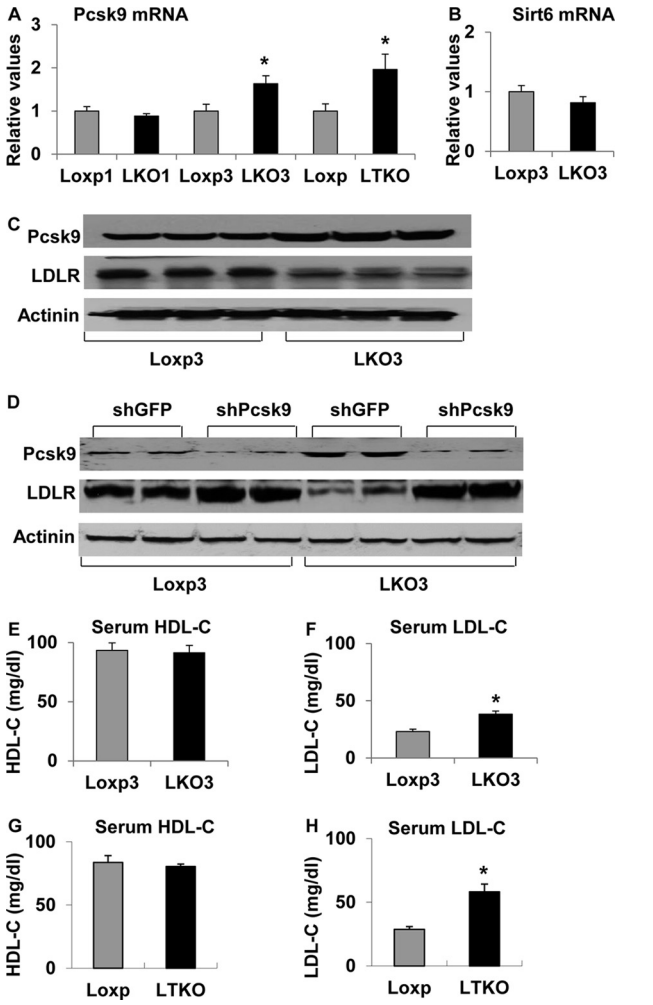

FIGURE 3. **Pcsk9 gene expression and LDL-cholesterol were elevated in FoxO3 liver-specific knock-out mice.** *A*, real-time PCR analysis of Pcsk9 mRNAs in the livers of control, LKO1 (FoxO1 liver-specific knock-out), LKO3 (FoxO3 liver-specific knock-out), and LTKO (FoxO1/3/4 liver-specific knock-out) mice ($n = 4–8$). Control values were normalized to 1. *B*, Sirt6 mRNAs were analyzed by real-time PCR in the livers of control and LKO3 mice ($n = 4$). *C*, Western blot analysis of Pcsk9 and LDLR proteins in control and LKO3 mouse livers. *D*, immunoblot analysis of Pcsk9 and LDLR in mouse primary hepatocytes transduced with shGFP or shPcsk9 adenoviruses. *E–H*, serum HDL-C and LDL-C were measured in control floxed, LKO3, and LTKO mice ($n = 6–8$). Data are the mean ± S.E.; *, $p \leq 0.05$ by *t* test.

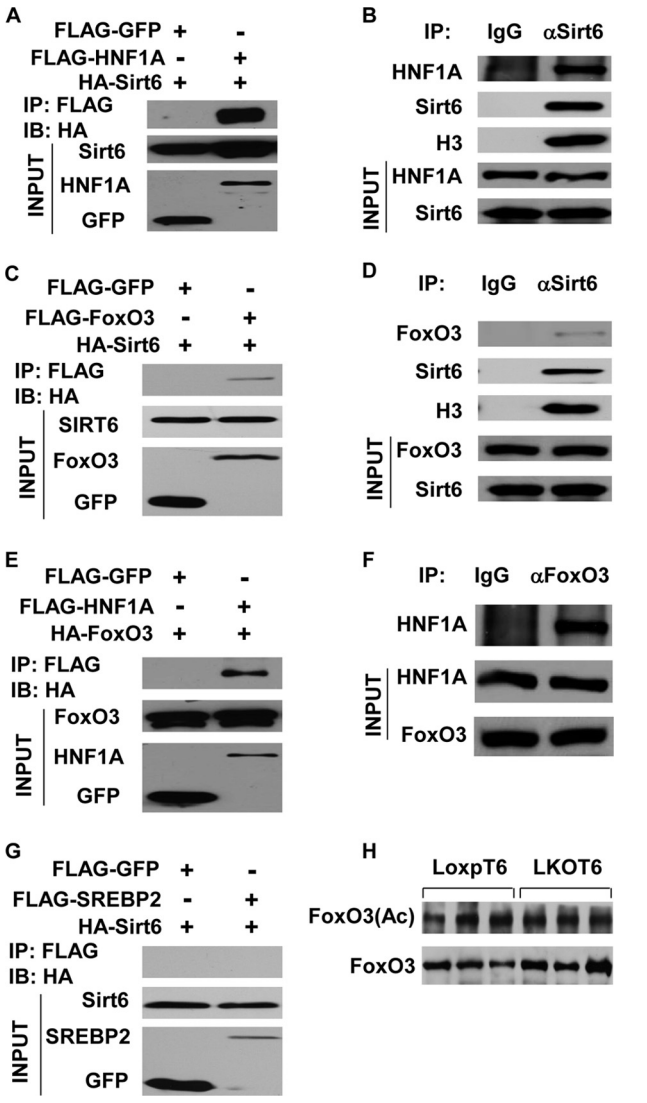

FIGURE 4. **Sirt6 interacts with HNF1A and FoxO3.** *A*, co-IP analysis of a potential interaction between Sirt6 and HNF1A by transfection of corresponding DNA plasmids into HEK293 cells. *IB*, immunoblot. *B*, the Sirt6-HNF1A interaction was verified in mouse primary hepatocytes by immunoprecipitation using Sirt6 antibodies. A positive control histone H3 was also included. *C*, co-IP analysis of a possible interaction between Sirt6 and FoxO3 in HEK293 cells. *D*, the Sirt6-FoxO3 interaction was validated in mouse primary hepatocytes by immunoprecipitation with Sirt6 antibodies. Histone H3 was also analyzed in the IP. *E*, co-IP analysis of a potential interaction between FoxO3 and HNF1A in HEK293 cells. *F*, immunoprecipitation analysis of the FoxO3-HNF1A interaction in mouse primary hepatocytes using FoxO3 antibodies. *G*, co-IP analysis indicates no interaction between Sirt6 and SREBP-2 in HEK293 cells. *H*, FoxO3 acetylation analysis of control and LKOT6 liver lysates by immunoprecipitation using FoxO3 antibodies and immunoblotting with anti-acetyl lysine antibodies.

could interact with HNF1A and FoxO3 but not SREBP-2 (Fig. 4, *A–D* and *G*). We also observed an interaction between FoxO3 and HNF1A in HEK293 cells and mouse primary hepatocytes (Fig. 4, *E* and *F*). To examine whether Sirt6 has any effect on FoxO3 acetylation, we carried out immunoprecipitation and immunoblot analyses of FoxO3 acetylation in control and LKOT6 liver lysates. The data indicated that Sirt6 deficiency did not have any significant effect on the overall acetylation of FoxO3 (Fig. 4*H*).

To assess whether Sirt6 could bring about epigenetic changes to the *Pcsk9* gene promoter, we overexpressed either GFP or Sirt6 in mouse primary hepatocytes and subsequently performed ChIP analysis of Sirt6 association and histone H3 acetylation. The results showed that Sirt6 was highly enriched at the 5′-UTR of the *Pcsk9* gene, and H3K9 and H3K56 acetylation levels were dramatically decreased in Sirt6 overexpressed hepatocytes (Fig. 5, *A* and *B*). Conversely, those histone modifications were elevated in the liver of LKOT6 mice (Fig. 5*C*). These data suggest that Sirt6 may be involved in H3K9 and H3K56 modifications in the *Pcsk9* gene because Sirt6 is known to deacetylate both sites (44–47). Because Sirt6 could interact with FoxO3, we also performed ChIP analysis of histone acetylation in FoxO3 overexpressed or knock-out hepatocytes. Similar to Sirt6 overexpression, FoxO3 overexpression also remarkably reduced acetylation of H3K9 and H3K56 (Fig. 5*D*). FoxO3 deficiency not only dramatically reduced association of Sirt6 with the 5′-UTR of the *Pcsk9* gene but also led to an increase of acetylation of H3K9 and H3K56 when endogenous

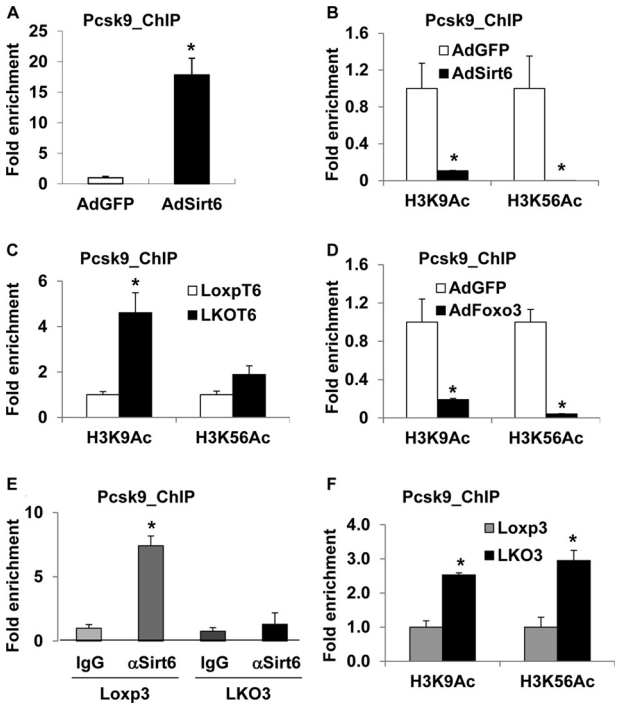

FIGURE 5. **FoxO3 and Sirt6 modulate histone acetylation in the chromatin of the *Pcsk9* gene.** *A* and *B*, association of Sirt6 with the 5′-UTR chromatin of the *Pcsk9* gene and histone H3 acetylation in the same region were analyzed by ChIP in mouse primary hepatocytes transduced with GFP and Sirt6 adenoviruses. Data are shown as -fold enrichment relative to the GFP control. *C*, the acetylation levels of H3K9 and H3K56 were analyzed by ChIP in the 5′-UTR of the *Pcsk9* gene in control and LKOT6 livers. Data are presented as -fold enrichment relative to the LoxpT6 control. *D*, the effect of FoxO3 overexpression on histone acetylation in the 5′-UTR chromatin was analyzed using ChIP in mouse primary hepatocytes transduced with GFP- or FoxO3-expressing adenoviruses. Data are expressed as -fold enrichment relative to the GFP control. *E* and *F*, ChIP analysis of Sirt6 association with the 5′-UTR of the *Pcsk9* gene and histone H3 acetylation in control and LKO3 mouse primary hepatocytes using corresponding specific antibodies. Data in *panel E* are presented as -fold enrichment relative to the IgG control in the Loxp3 group, and data in *panel F* are shown as -fold enrichment relative to the Loxp3 control. Data are the mean ± S.E.; *, $p \leq 0.05$ by *t* test.

protein antibodies were used for the ChIP analyses (Fig. 5, *E* and *F*).

Sirt6 Overexpression Lowers LDL-cholesterol in High Fat Diet-treated Mice—To examine a potential role of Sirt6 in the protection against hypercholesterolemia, we first analyzed *Sirt6* gene expression in the liver of mice treated with a high fat diet (HFD) for 2 months. The hepatic levels of Sirt6 mRNA and protein were decreased in the liver of HFD-fed mice as compared with chow diet group (Fig. 6, *A* and *B*). To test whether overexpression of Sirt6 could improve hypercholesterolemia, we injected GFP- or Sirt6-expressing adenoviruses into the HFD-treated mice. Two weeks later, we analyzed hepatic *Pcsk9* and *Ldlr* gene expression and serum cholesterol. HFD induced *Pcsk9* mRNA and protein levels, and Sirt6 overexpression significantly suppressed the *Pcsk9* gene expression (Fig. 6, *B–D*). With regard to LDLR, HFD induced expression of the *Ldlr* gene, and Sirt6 overexpression further increased the LDLR protein levels (Fig. 6, *B–D*). Whereas HDL-cholesterol levels were not changed, total and LDL-cholesterol levels were significantly decreased in the Sirt6-overexpressed mice relative to control mice on HFD (Fig. 6*E*). These data reinforce the notion that Sirt6 plays a critical role in the LDL-cholesterol homeostasis.

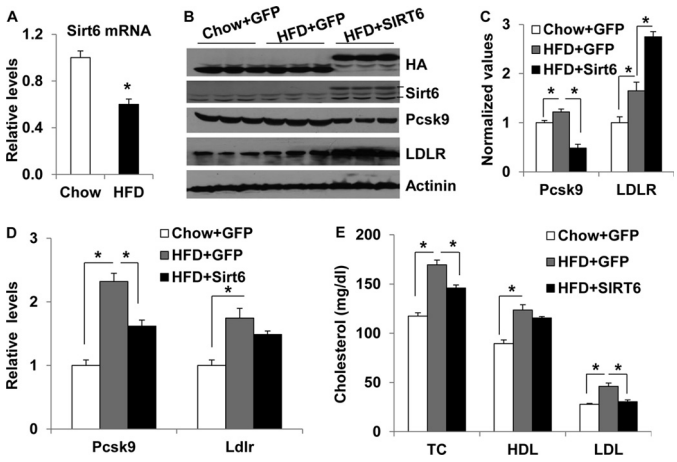

FIGURE 6. **Sirt6 overexpression reduces *Pcsk9* gene expression and serum LDL-cholesterol.** *A*, Sirt6 mRNA levels in the liver of wild-type mice ($n = 5–8$) fed chow or 2-month HFD were analyzed by real-time PCR. *B*, Western blot analysis of liver proteins from chow or HFD-treated wild-type mice infected with GFP or Sirt6 adenoviruses. *C*, quantitative analysis of Pcsk9 and LDLR proteins in *panel B*. The immunoblot data were quantified by the Quantity One software (Bio-Rad) and normalized to the loading control actinin. *D*, Pcsk9 and Ldlr mRNAs in the liver of chow or HFD fed mice ($n = 5–8$) that were infected with GFP or Sirt6 adenoviruses were analyzed by real-time PCR. *E*, serum cholesterol measurements in the chow and HFD-fed mice overexpressed GFP or Sirt6 ($n = 5–8$). *TC*, total cholesterol. Data are the mean ± S.E.; *, $p \leq 0.05$ by *t* test.

DISCUSSION

In this work we have demonstrated that hepatic Sirt6 and FoxO3 have an important role in the regulation of LDL-cholesterol homeostasis. Because Sirt6 is decreased in the livers of obese animals and humans (38, 48), it implicates a potential consequence for the development of hypercholesterolemia, particularly high LDL-cholesterol. Previously, it was reported that systemic overexpression of Sirt6 in mice can lower LDL-cholesterol under conditions of either chow or high fat diet; however, the underlying mechanism is not clear (49). According to our data, we speculate that down-regulation of the *Pcsk9* gene expression may be responsible for the low LDL-cholesterol phenotype in the *Sirt6* transgenic mice. Additionally, Sirt6 also significantly represses fatty acid and cholesterol biosynthetic genes and activates fatty acid oxidation genes (38, 43). Apparently, Sirt6 has a salutary effect on lipid homeostasis.

Because Sirt6 is an NAD-dependent deacetylase and mainly targets to histone H3, it may normally be recruited by transcription factors for regulation of specific genes. With regard to the *Pcsk9* gene, SREBP-1/2 and HNF1A have been shown to play significant regulatory roles (29, 31, 33, 34, 36). Our data suggest that Sirt6 may be recruited by FoxO3 to the *Pcsk9* gene promoter to suppress the gene expression. FoxO transcription factors are known to have both positive and negative effects on gene regulation. The negative effects of FoxOs can be mediated by several different mechanisms, including displacement of regulatory cofactors, recruitment of co-repressor or histone deacetylase, sequestration of other transcription factors, or promotion of associated protein degradation (50). In the case of *Pcsk9* gene regulation, our data suggest that FoxO3 may suppress the HNF1A transcriptional activity on the *Pcsk9* gene promoter by displacing this transcription factor and recruiting the histone deacetylase Sirt6 as well. This regulation may occur

during starvation because under that condition Sirt6 and FoxO3 are both active. As a result of the Sirt6 recruitment, deacetylation of H3K9 and H3K56 by Sirt6 creates a repressive state in the chromatin of the *Pcsk9* gene promoter to suppress the gene transcription. Additionally, reduced levels of SREBPs and HNF1A may also contribute to the down-regulation of the *Pcsk9* gene during fasting (27, 29, 33). Upon feeding, the activity of FoxO3 and Sirt6 is decreased, and the levels of nuclear SREBPs are increased; the *Pcsk9* gene transcription is thus activated. With regard to the involvement of FoxOs in the regulation of the *Pcsk9* gene, some questions remain to be addressed in the future. First, why does FoxO3 play a major role rather than FoxO1, as FoxO1 is also highly abundant in the liver as well? In agreement with our data, previous reports have also shown that FoxO1 does not play a significant role in LDL-cholesterol regulation (36, 51, 52). Second, what is the role of FoxO3 in the regulation of the *Pcsk9* gene in obese and diabetic conditions? Whereas several reports have shown that feeding or insulin can induce the *Pcsk9* gene expression (23, 29, 53), another one has documented an increase in the *Pcsk9* gene expression in the liver of insulin receptor knockdown mice (36). In insulin-deficient type 1 diabetic rats, hepatic Pcsk9 mRNAs are dramatically decreased (53); however, in ob/ob leptin-deficient obese mice, hepatic Pcsk9 mRNAs are also decreased by 2-fold (36). Further investigation is needed to clarify what causes differential regulation of the *Pcsk9* gene expression under those conditions.

Recently, we have reported that FoxO3 and Sirt6 also suppress the *Srebp2* gene expression in the liver (43). This suggests that both factors may have a coordinated role in cholesterol homeostasis. By regulating the *Srebp2* gene, the master regulator of cholesterol biosynthesis, FoxO3 and Sirt6 have an impact on total cholesterol levels in the circulation. With fine-tuning on the *Pcsk9* gene expression, Sirt6 and FoxO3 enhance the salutary effects by lowering LDL-cholesterol levels.

As Pcsk9 plays an important role in LDL-cholesterol homeostasis, proper regulation of the *Pcsk9* gene expression by Sirt6 and FoxO3 may contribute to cardiovascular health of organisms. It is known that both Sirt6 and FoxO3 are associated with longevity in mammals (47, 54–59). Thus, it should be interesting to look into how Sirt6 and FoxO3 may influence longevity through regulation of LDL-cholesterol.

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